FUNGAL ECOLOGY 6 (2013) 339–355

available at www.sciencedirect.com

SciVerse ScienceDirect

journal homepage: www.elsevier.com/locate/funeco

The gut of Guatemalan passalid beetles: a habitat colonized by cellobiose- and xylose-fermenting yeasts

Hector URBINA[a,*], *Jack SCHUSTER*[b], *Meredith BLACKWELL*[a]

[a]*Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803, USA*
[b]*Laboratorio de Entomología Sistemática, Universidad del Valle de Guatemala, Cuidad de Guatemala, Apartado 82, Guatemala*

ARTICLE INFO

Article history:
Received 2 May 2013
Revision received 29 May 2013
Accepted 31 May 2013
Available online 9 August 2013
Corresponding editor:
Fernando Vega

Keywords:
Central America
Digestive system
Insect
Polyphaga
Saccharomycotina
Scarabaeoidea

ABSTRACT

The gut of insects is a productive environment for discovering undescribed species of yeasts, and the gut of wood-feeding insects of several families is especially rich in yeasts that carry out the fermentation of cellobiose and xylose. Passalid beetles (Passalidae, Coleoptera) live in dead wood that they ingest as their primary food source. We report the isolation, molecular identification and physiological characterization of 771 yeast cultures isolated from the gut of 16 species of passalids collected in nine localities in Guatemala. Ascomycete yeasts were present in the gut of every passalid studied, and the xylose-fermenting (X-F) yeasts *Scheffersomyces shehatae* and *Scheffersomyces stipitis* were the most abundant taxa isolated. The gut of the beetles also contained undescribed cellobiose-fermenting and X-F species in the *Lodderomyces*, *Scheffersomyces* and *Spathaspora*, and undescribed species in *Sugiyamaella* clades as well as rare yeast species in the *Phaffomyces* and *Spencermartinsiella* clades. Basidiomycete yeasts in the genera *Cryptococcus* and *Trichosporon* were also common. The yeast species richness was influenced by the host species and the substrate, and gut-inhabiting yeasts have the ability to survive the differing physiological conditions of several gut compartments.

Introduction

Yeast isolations from the gut of insects have led to discovery of a large number of new species of yeasts (Suh et al., 2003, 2004a, 2005a, 2006; Suh and Blackwell, 2005, 2006; Berkov et al., 2007; Rivera et al., 2009; Grunwald et al., 2010; Houseknecht et al., 2011; Calderon and Berkov, 2012; Urbina et al., 2013). Despite these efforts the diversity of gut-inhabiting yeasts remains understudied. Suh et al. (2005a) suggested that only 50 % of the gut-inhabiting yeasts from certain Panamanian mushroom-feeding beetles have been discovered after extensive sampling from more than 20 insect families. The new yeasts are important because they fill taxon-sampling gaps, help to understand the phylogenic relationships among members of Saccharomycotina, and synthesize enzymes, vitamins and other products that could be useful in biotechnological processes.

Recent rising fuel costs have stimulated the search for new yeasts capable of fermenting cellobiose and D-xylose, which can be used in the production of bioethanol. Many of the yeasts discovered were isolated from soil, plant tissues and the gut

* *Corresponding author*. Tel.: +1 (225) 603-0534; fax: +1 (225) 578 2597.
E-mail address: hurbinay@gmail.com (H. Urbina).
1754-5048
http://dx.doi.org/10.1016/j.funeco.2013.06.005

of wood-feeding beetles (e.g. Cerambycidae, Curculionidae, Lucanidae, Passalidae) (Suh et al., 2003, 2005a; Zhang et al., 2003; Suh and Blackwell, 2004; Berkov et al., 2007; Cadete et al., 2009; Tanahashi et al., 2010; Santos et al., 2011; Calderon and Berkov, 2012). In general the characterization of yeasts associated with the gut of lignicolous insects confirmed the consistent association between xylose-fermenting (X-F) yeasts and insects.

Passalid beetles (Passalidae, Coleoptera) mostly feed on rotting wood and spend most of their lives inside rotting logs (Reyes-Castillo, 1970; Boucher, 2005; Schuster, 2006). The beetle family comprises approximately 960 species, and in the most recent taxonomic reclassification of the family, besides their distribution, external morphological characters and biology, the macro-morphology of the anterior hindgut is another character proposed to distinguish the five subfamilies in Passalidae (Fonseca et al., 2011). The subfamilies Passalinae and Proculinae are distributed exclusively in the New World, while Aulacocyclinae, Macrolininae and Solenocyclinae occur in Asia, Africa and Australia (Boucher, 2005; Fonseca et al., 2011).

The majority of passalids exhibit subsocial behavior that includes parental care by the feeding of a mixture of digested wood and feces to larvae and juveniles. Adults also envelop the larvae at the time of metamorphosis to pupae with a covering of frass and pre-digested wood, the pupal chamber, that becomes the first meal for juveniles as they emerge (Reyes-Castillo, 1970; Tallamy and Wood, 1986). Such behavior suggests that horizontal transfer of microbes is required for the complete metamorphosis from larvae to adults, and apparently larvae cannot survive when fed only sterilized pulverized rotten wood (Pearse et al., 1936; Reyes-Castillo, 1970; Nardi et al., 2006; Berkov et al., 2007; Rao et al., 2007). These findings emphasize the important role of the passalid gut microbiota in the digestion of the substrate in wood-feeding insects.

The biology, ecology and physiology of passalids are best studied for *Odontotaenius disjunctus*, a common species in the southeastern United States (Pearse et al., 1936; Roberts, 1952; Bryan, 1954; Hiznay and Krause, 1955; Ferguson and Land, 1961; Robertson, 1962; Collings, 1966; Burnett et al., 1969; Ward, 1971; Delfinado and Baker, 1975; Dismukes and Mason, 1975; Schuster, 1975; Gibson, 1977; Rains and Dimock, 1978; Buchler et al., 1981; Mason et al., 1983; Tafuri and Tafuri, 1983; Wit et al., 1984; Sawvel et al., 1992; MacGown and MacGown, 1996; King and Fashing, 2007; Punzo, 2007; Jackson et al., 2009; Wicknick and Miskelly, 2009). Nardi et al. (2006) characterized the morphological and cellular transformations of the hindgut region of the digestive system from larva to adult of *O. disjunctus*, and described the physical distribution and arrangement of the microbiota in the gut compartments.

The digestive system of an adult *O. disjunctus* is often over 10 cm long and a complex organ, at least twice as long as the length of an individual. The three regions of the gut (foregut, midgut and hindgut) are conspicuously differentiated and, in addition, the hindgut has readily distinguishable anterior and posterior compartments (Nardi et al., 2006). The gut regions also differ in their physiological conditions of O_2, CO_2 and pH (Ceja-Navarro et al., 2013). Another structure, a conspicuous diverticulum, is present at the anterior end of the hindgut. In addition to bacteria and yeasts, trichomycetes, amoebae, nematodes, flagellated protists and filamentous fungi may be present in the gut of *O. disjunctus*. Most notable, a variety of bacteria attach to form conspicuous surface films in the anterior hindgut, while filamentous yeasts almost exclusively colonize the posterior hindgut attached by a holdfast (Suh et al., 2003; Nardi et al., 2006; Ceja-Navarro et al., 2013).

The ascomycete yeasts inhabiting the gut of *O. disjunctus* have been characterized by molecular and physiological means. Previous studies have confirmed the predominant and consistent presence of the X-F yeast *Scheffersomyces stipitis* and other less abundant X-F yeasts including *Scheffersomyces shehatae*, *Candida maltosa* (*Lodderomyces* clade) (Suh et al., 2008) and the cellobiose-fermenting (C-F) yeast *Scheffersomyces ergatensis* (Suh et al., 2003; Zhang et al., 2003; Nardi et al., 2006). The X-F yeasts *Spathaspora passalidarum* and *Candida jeffriesii* (*Spathaspora* clade) (Nguyen et al., 2006), the trehalose-fermenting yeast *Kazachstania intestinalis* (Suh and Zhou, 2011), the D-xylose-assimilating yeast *Sugiyamaella bullrunensis* (Houseknecht et al., 2011) and the basidiomycete yeast *Trichosporon xylopini* (Gujjari et al., 2011) were all first described in association with *O. disjunctus*. The gut of this single passalid species has been a source for isolation of at least nine previously unknown ascomycete and basidiomycete yeasts that exhibit the ability to utilize several wood components.

Only a few studies have characterized yeasts from the gut of other passalids such as *Paxillus leachi*, *Passalus interstitialis*, *Ptichopus angulatus, Verres hageni*, *Verres sternbergianus* and *Veturius platyrhinus*, which occur in Panama and Peru. Species closely related to *Candida mycetangii* (*Wickerhamomyces* clade) and *S. stipitis*, as well as other yeasts including *Candida parapsilosis* (*Lodderomyces* clade), *Candida temnochilae* (*Yamadazyma* clade) and the basidiomycete yeast *Trichosporon insectorum* were isolated from these beetles (Suh et al., 2003, 2005a,b; 2008; Fuentefria et al., 2008). The diversity of yeasts associated with the gut of most passalid beetles is, however, poorly known considering the worldwide distribution of the group.

The principal aim of this study was to expand the knowledge of diversity of ascomycete and basidiomycete yeasts in the gut of Guatemalan passalids. This aim was achieved through the isolation, identification and characterization of 771 yeast isolates from the gut of 16 species of passalids collected at nine localities in Guatemala. Our results confirm the association of passalids and X-F yeasts, including undescribed species in the *Phaffomyces*, *Scheffersomyces*, *Spathaspora*, *Spencermartinsiella* and *Sugiyamaella* clades.

Materials and methods

Passalid collection

A total of 47 adult specimens identified as 16 species of passalids in two subfamilies, Passalinae and Proculinae were collected at nine localities in Guatemala (Fig 1, Tables 1 and 2). The localities were selected based on the endemic passalid zones described previously (Schuster, 1991, 1992a,b, 1993, 2002, 2006; Schuster et al., 2000, 2003). Targeted searches in passalid galleries in rotted logs were effective for collecting passalid individuals, and only one passalid specimen was collected at a light trap.

Fig 1 – Collecting sites indicated on map of Guatemala (numbers correspond with numbers of sites in Table 1).

Beetle sampling

Five individuals per species per site were selected for dissection. Each beetle individual was surface disinfected by washing in 70 % ethanol (5 min), 5 % bleach (5 min) and sterile water (10 min) prior to dissection. To carry out gut removal, each specimen was placed dorsal side up on a flame-sterilized glass slide, and sterile forceps were used to remove the elytra to allow access to the gut (Fig 2).

All dissections were performed the same day or 1 d following collection; until dissection, beetles were kept alive in plastic containers with the rotted wood from which they were collected. All dissected individuals were preserved immediately in absolute ethanol for identification (Reyes-Castillo, 1970; Schuster, 1975, 1992a,b, 1993; 2002; Boucher, 2005; Fonseca et al., 2011).

Yeast isolation and culture

The gut of each beetle was dissected into three regions: foregut–midgut (F–M) and anterior (AHG) and posterior hindgut (PHG) (Fig 2). The F–M regions were maintained as one to preserve the head that contains morphological characters used for identification. Each gut region was homogenized separately using a sterile plastic pestle in 500 µl of 0.7 % saline solution with 0.01 % Tween 80. Next, 100 µl of each homogenized region was superficially plated on YPDM medium (0.3 % yeast extract, 0.5 % Bacto peptone, 1 % dextrose, 0.3 % malt extract and 2 % agar)

Table 1 – Guatemalan collecting sites and passalid endemic zone classification (PEZC) recognized by Schuster (2006)

Site	Locality	Department	Vegetation type	Latitude	Longitude	Altitude (masl)	PEZC
1	Carmona Mountain	Sacatepéquez	Tropical forest	N14°32′29″	W90°42′04″	1 910	4a
2	Purulha	Baja Verapaz	Cloud forest	N15°12′27″	W90°13′18″	1 950	–
3	Puerta Parada	Guatemala	Secondary forest	N14°33′23″	W90°27′47″	1 843	–
4	Cementos El Progreso	El Progreso	Dry forest	N14°48′52″	W90°16′38″	1 080	–
5	Pueblo Nuevo Viñas	Santa Rosa	Secondary forest	N14°12′17″	W90°30′20″	1 641	7c
6	Miramundo Mountain	Escuintla	Secondary forest	N14 13′39″	W90 28′42″	1 308	7b
7	El Trifinio	Chiquimula	Cloud forest	N14°27′01″	W89°23′22″	1 312	6
8	La Union	Zacapa	Secondary forest	N14 58′26″	W89 17′41″	1 150	4b
9	San Gil Mountain	Izabal	Rain forest	N15°38′55″	W88°48′52″	389	–

Table 2 – Passalid beetles collected in Guatemala. Total number of species (*) and endemic species (Δ) reported for Guatemala were obtained form Schuster (2006)

Host	Collecting sites: Carnoma Mountain (1)	Purulha (2)	Puerta Parada (3)	Cementos El Progreso (4)	Pueblo Nuevo Viñas (5)	Miramundo Mountain (6)	El Tifrinio (7)	La Union (8)	San Gil Mountain (9)	Number of individuals dissected	Occurrence of species per site
Subfamily Passalinae (4 species collected)											
Passalus sp.			1							**1**	**1**
Passalus interstitialis				1						**1**	**1**
Passalus punctatostriatus				3	3					**6**	**2**
Passalus punctiger									2	**2**	**1**
Subfamily Proculinae (12 species collected)											
Arrox agassizi				1						**1**	**1**
Chondrocephalus debilis	1	1			1					**3**	**3**
Chondrocephalus purulensis		1			1		1			**3**	**3**
Chondrocephalus sp.						1				**1**	**1**
Ogyges championi Δ		4								**4**	**1**
Ogyges hondurensis Δ					2					**2**	**1**
Ogyges laevissimus Δ	1									**1**	**1**
Oileus sargi	2				1	3	1	1		**8**	**5**
Popilius eclipticus									3	**3**	**2**
Proculus mnizechii								1		**1**	**1**
Vindex sp.							4			**4**	**1**
Xylopassaloides chortii Δ								1		**1**	**1**
Total individuals collected per site	**4**	**6**	**1**	**5**	**8**	**4**	**6**	**3**	**5**	**42**	–
Total passalid species collected per site	**3**	**3**	**1**	**3**	**5**	**2**	**2**	**3**	**2**	–	**24**
Total passalids reported*	**6**	**12**	**2**	**13**	**7**	**2**	**12**	**10**	**6**	–	**75**

The passalid subfamilies are in bold.

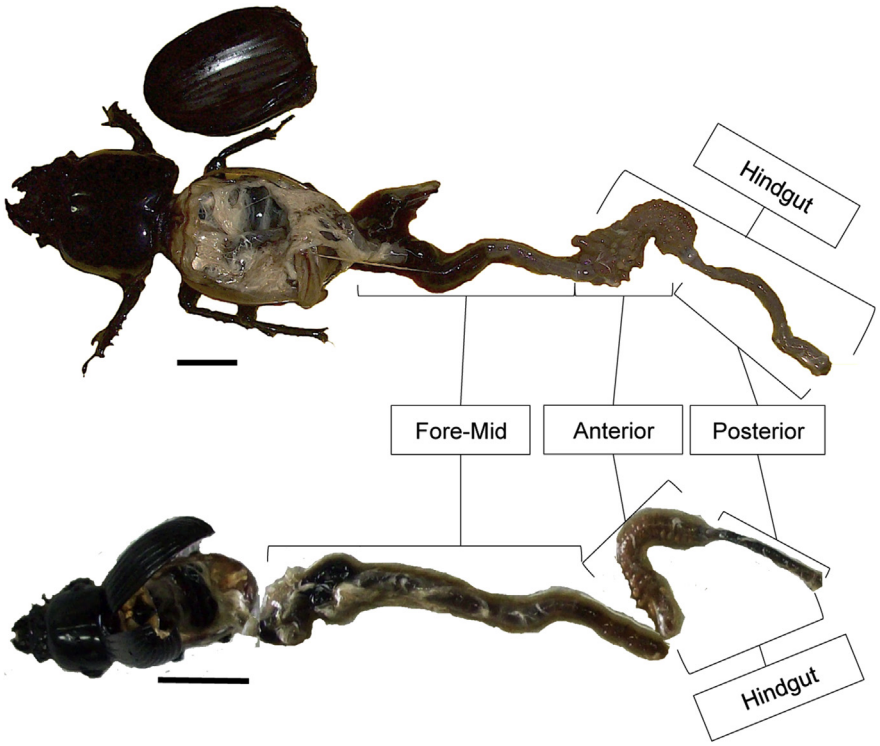

Fig 2 – Gut dissection of the passalids *Proculus mnizechi* (above) and *Chondrocephalus purulensis* (below); bar = 1 cm.

supplemented with 0.05 % calcium carbonate, vitamins and salts (Yarrow, 1998).

The YPDM medium was acidified with 0.6 ml of concentrated HCl l^{-1} and 0.35 μg l^{-1} chloramphenicol was added to reduce bacterial growth as was previously described (Urbina et al., 2013). After 3 d of incubation at room temperature, 12 yeast colonies per gut region were selected, resulting in 36 yeast strains per individual beetle whenever possible. The following data from the 771 yeast strains was documented: collecting date (year, month and day), site (1–9, as numbered in Table 1), log sample (consecutive numbers given on each collecting day), beetle individual (consecutive numbers given on each collecting log), gut region from which each yeast strain was isolated (F–M = 1, AHG = 2 and PHG = 3) and yeast isolate (consecutive numbers given to each colony). Cultures are stored in 15 % glycerol at −80 °C at (The Louisiana State University Museum of Natural Sciences, Department of Biological Sciences, Louisiana State University, Baton Rouge, Louisiana, United States) and at Micoteca Rubén Mayorga Peralta – MICG (Departamento de Microbiología, Escuela de Química Biológica, Facultad de Ciencias Químicas y Farmacia, Universidad de San Carlos de Guatemala, Guatemala City, Guatemala).

Other yeast culture techniques and physiological characterizations were carried out following the methods of Yarrow (1998), Barnett et al. (2000) and Kurtzman et al. (2011).

Molecular studies

Genomic DNA was extracted using a Wizard® Genomic DNA purification kit (Promega). The concentration, integrity and purity of total extracted DNA were verified by gel electrophoresis in 0.8 % agarose in 0.5 × Tris–Borate–EDTA (TBE) buffer. Rapid molecular identification was performed by PCR amplification of the D1/D2 region of the large subunit (LSU) of the rRNA gene (rDNA) (~600 bp) (Kurtzman and Robnett, 1998; Kurtzman and Suzuki, 2010).

PCR amplifications of the internal transcribed spacers 1 and 2 (ITS ~ 600 bp) and the small subunit locus (SSU ~ 1.6 Kbp) of the rDNA were carried out in addition to the LSU locus to increase the robustness of the phylogenetic analyses and the accuracy of species identification (Robbertse et al., 2006; Schoch et al., 2009, 2012). The SSU was amplified using the combination of primers NS1 (forward) (5′-GTAGTCATATGCTTGTCTC-3′) and NS8 (reverse) (5′-TCCGCAGGTTCACCTACGGA-3′); ITS-LSU loci were amplified using the combination of primers ITS1 (forward) (5′-TCCGTAGGTGAACCTGCGG-3′) and LR3 (reverse) (5′-CCGTGTTTCAAGACGGG-3′) in a PCR reaction with 20 μg of total DNA, 0.5 mM DTPs, 2.5 mM $MgSO_4$, 1 × Colorless GoTaq® Flexi Buffer (Promega) and 1 U of Taq polymerase (Promega) in 25 μl of final volume. The PCR amplification program included 5 min of DNA pre-denaturing at 95 °C, followed by 35 cycles of 1 min of DNA denaturing at 95 °C, 45 s of primer annealing at 55 °C and 2 min of extension at 72 °C and 10 min of final PCR extension. The purified PCR products were sequenced in both directions by Beckman Coulter Genomics (Danvers, MA). Sequences were deposited in GenBank with the following accession numbers: SSU (JQ008831–JQ008930), ITS (JN831680–JN831706) and LSU (JN804869–JN805539).

Phylogenetic analyses

Contiguous sequences and sequencing manipulations were performed in Se-AL v2.01a11 (available at http://tree.bio.ed.ac.uk/software/seal/) and MESQUITE v2.74 (Maddison and Maddison, 2005). The sequence alignments were carried out using the online interface MAFFT v6.859 (http://mafft.cbrc.jp/alignment/software/) with different advanced alignment strategies per locus: LSU, global homology (G-INS-i); ITS, one conserved domain (L-INS-i) and SSU, secondary structure of RNA (Q-INS-i). ITS loci were realigned using the software SATé v2.1.2 (Liu et al., 2012) and the ambiguous sequence alignment ends were eliminated. Maximum likelihood (ML) phylogenetic inference was performed in RAxML-VI-HPC (Stamatakis, 2006) using a partitioned multilocus matrix under a general time reversible model with a gamma distribution of among site rate variation (GTRGAMMA). Maximum likelihood support was estimated using 1 000 bootstrap replicates. Tree editing was done using the software FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

Statistical analyses

A rarefaction curve was performed with the software EstimateS v8.2.0 (Colwell, 2006) using a presence/absence matrix by yeast species per individual passalid. We used the non-parametric species richness index Chao 2 (Chao, 1987) estimator that has been suggested as the most suitable to predict the number of species in continuing fungal surveys (Unterseher et al., 2008). The rarefaction curve obtained was adjusted using the Clench model under the non-linear simplex and quasi-Newton estimator implemented in the software STATISTICA v10. (http://www.statsoft.com) recommended by previous researchers (Soberon and Llorente, 1993; Gotelli and Colwell, 2001; Jimenez-Valverde and Hortal, 2003).

The following analyses were performed in R v2.15.0 (R Development Core Team, 2005) and only the species of passalids yielding more than 60 yeast strains were included in the analyses to avoid variation due to small sample size (Table 3). The Kruskal–Wallis (H) non-parametric test was used to determine differences in yeast composition between individuals and species of passalids and among gut regions. To investigate which variables affect the distribution of yeasts across the Guatemalan passalids a correlation analysis was performed between the following variables: yeast species, host data (host individual, length, width, length/width ratio and subfamily), type of ecosystem (dry, cloud, or wet forest), collecting data (altitude, longitude, latitude, locality and rotten log). Among the variables, rotten log, individual passalid and locality were highly correlated so log and locality were omitted for the next analyses. The data-driven model building approach *forward selection* was computed with the library *packfor* (Oksanen et al., 2012) with alpha = 0.05 and 1 000 permutations using yeast species richness and variable factors datasets, both arranged by passalid individual. The diversity indexes were computed using the library *vegan* (Oksanen et al., 2012).

Results and discussion

Diversity of cellobiose- and xylose-fermenting yeasts among Guatemalan passalids

The presence of ascomycete yeasts in the gut of Guatemalan passalids was confirmed in all individuals dissected. The 771 yeast strains isolated corresponded to approximately 78 species based on nucleotide differences in the SSU, LSU and ITS molecular markers and their phylogenetic relatedness. The most abundant Saccharomycotina lineages among Guatemalan passalids were *Scheffersomyces* and *Spathaspora* (Debaryomycetaceae) (560 isolates, approximately 76.5 %) (Figs 3 and 4). The X-F yeasts *S. shehatae* (314 isolates, 42.9 %) and *S. stipitis* (109 isolates, 14.9 %) were the most common ascomycete yeasts identified. These findings are in agreement with previous studies, which reported both yeasts as common gut-inhabiting microbes in lignicolous insects (e.g. Suh et al., 2003; Zhang et al., 2003; Berkov et al., 2007; Grunwald et al., 2010; Urbina et al., 2013). These findings differed from those of previous studies because the most common X-F gut yeast from Guatemalan beetles was *S. shehatae* instead of *S. stipitis* that is abundant in the gut of the USA *O. disjunctus* with *S. shehatae* isolated only occasionally (Meredith Blackwell, unpublished data). The major changes in the yeast community composition of passalids might be driven by a number of factors including plant diversity, geographical distribution, passalid diversity and climatic conditions.

The X-F yeast *S. shehatae* previously was described as a species complex of insect-associated yeasts isolated from several families of wood-feeding insects and rotted wood collected from the Netherlands, France, Canada, South Africa, Germany, Chile (http://www.cbs.knaw.nl) and the United States (http://nrrl.ncaur.usda.gov). Recently, additional species

Table 3 – Gut-inhabiting yeasts from different passalid species

	P. punctatostriatus	*C. debilis*	*C. purulensis*	*O. sargi*	*Vindex* sp.	All hosts
Number of sampled individuals	6	3	4	8	4	25
Yeast species richness per host spp.	19	19	22	21	21	60
Simpson's diversity index	0.95	0.95	0.95	0.95	0.95	–
Shannon–Wiener index	2.94	2.94	3.09	3.04	3.09	–
Chao	131.5 ± 127.06	35.90 ± 12.40	94.25 ± 62.53	63.67 ± 33.24	64.67 ± 33.24	101.29 ± 19.07
Jackknife	31.50 ± 7.56	27.67 ± 8.97	34.75 ± 9.20	35.00 ± 7.12	34.00 ± 8.03	92.58 ± 9.92
Bootstrap	24.14 ± 3.62	23.04 ± 5.00	27.51 ± 4.40	26.50 ± 3.29	27.26 ± 3.80	74.16 ± 5.12
Sørensen index of dissimilarity	0.80	0.64	0.62	0.84	0.67	0.79

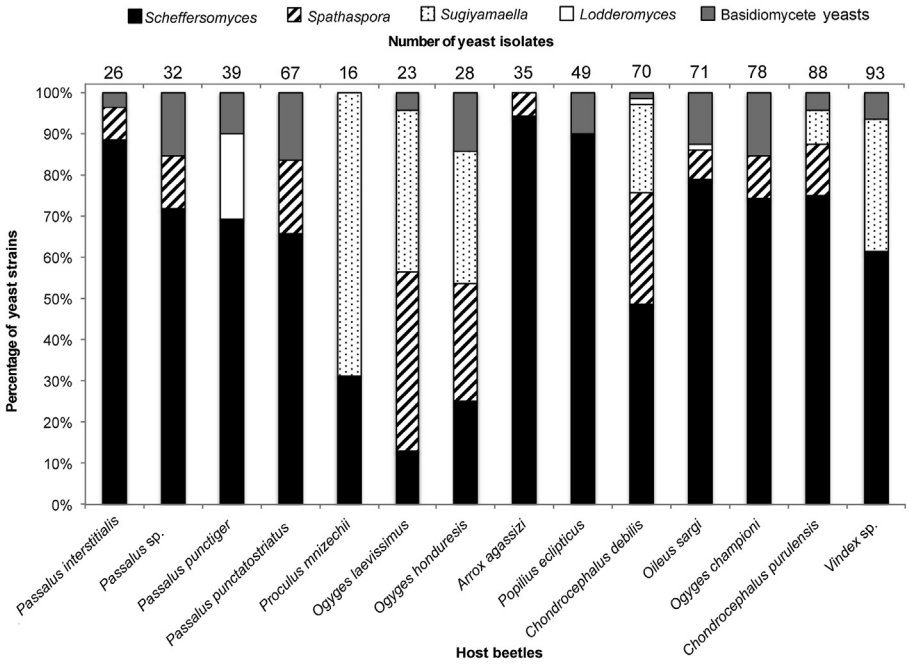

Fig 3 – Yeast clades isolated from the gut of Guatemalan passalid beetles. Subfamilies: Passalinae (*Passalus*) and Proculinae (*Arrox*, *Chondrocephalus*, *Ogyges*, *Oileus*, *Popilius* and *Vindex*).

closely related to *S. shehatae* (*Scheffersomyces virginianus*, *Scheffersomyces illinoinensis*, *Scheffersomyces quercinus* and *Scheffersomyces cryptocercus*) were discovered based on a multilocus phylogenetic analysis (Urbina and Blackwell, 2012; Urbina et al., 2013).

In agreement with several studies, the use of the LSU marker solely as a barcode is insufficient for the differentiation of all species in the *Scheffersomyces* clade (Kurtzman, 1990; Kurtzman and Robnett, 2007; Kurtzman and Suzuki, 2010). The newly recommended fungal barcode, the ITS marker (Schoch et al., 2012), was used to increase the accuracy of species discrimination among the *S. shehatae* isolates from the gut of Guatemalan passalids. Based on the characterization and the corresponding ability of most members to ferment D-xylose, at least eight undescribed species related to *S. shehatae* and *S. stipitis* were discovered in this study.

Urbina and Blackwell (2012) distinguished three subclades of *Scheffersomyces*: (1) the *S. stipitis* subclade, which is composed exclusively of X-F yeasts; (2) the *S. ergatensis* subclade of C-F yeasts; and (3) the *Scheffersomyces spartinae* subclade containing the marine yeast *S. spartinae* and recently described *Scheffersomyces gosingicus* from soil in Taiwan (Chang et al., 2011). Yeast species and undescribed strains from subclades *S. ergatensis* and *S. stipitis* were recovered from the gut of Guatemalan passalids, but no members of the *S. spartinae* subclade were isolated. These findings indicate that the gut of Guatemalan passalids is a suitable habitat for yeasts that ferment the wood components cellobiose and D-xylose.

Several undescribed species in the *Spathaspora* clade also were present in the gut of Guatemalan passalids. The *Spathaspora* clade comprises the X-F yeasts *C. jeffriesii*, *S. passalidarum* and *Spathaspora arborariae*, previously reported from the galleries of the passalid beetle *O. disjunctus* and rotten wood in Brazil (Nguyen et al., 2006; Barbosa et al., 2009), the xylose-assimilating yeasts *Candida insectamans*, *Candida xylanilytica*, *Candida materiae* and *Candida lyxosophyla*, isolated from wood in Asia, and the human pathogenic yeast *Candida subhashii* (van der Walt et al., 1972, 1987; Adam et al., 2009; Barbosa et al., 2009; Cadete et al., 2009; Boonmak et al., 2011). None of the yeasts previously classified in this clade was recovered from the gut of Guatemalan passalids and, therefore, all *Spathaspora* members isolated were undescribed species most closely related to *S. passalidarum*, *C. materiae* and *C. jeffriesii* (Figs 3 and 4) previously isolated in the Americas. Some of the undescribed *Spathaspora* yeasts also had the ability to ferment cellobiose and D-xylose, characteristics of some members of this clade (Fig 4).

Some *Lodderomyces* strains from the gut of Guatemalan passalids were also recovered (12 isolates, approximately 1.6 %), but, unlike *Scheffersomyces* or *Spathaspora* species, they were not consistently present among the host beetles (Fig 3). The *Lodderomyces* clade is a monophyletic group closely related to the *Spathaspora* and *Scheffersomyces* clades (Fig 4) and it contains the opportunistic human pathogenic yeasts *Candida albicans*, *Candida dubliniensis* and *Candida tropicalis*. *C. maltosa* and *C. tropicalis* are the only members of this clade that can ferment D-xylose (Lohmeier-Vogel et al., 1989; Lin et al., 2010). Suh et al. (2008) suggested an association between plant-feeding insects and *Lodderomyces* yeasts, based on the isolation of *C. tropicalis* from an unidentified passalid (Panama); *C. parapsilosis* from *V. hageni* and *P. angulatus* (Panama) and *C. maltosa* from *O. disjunctus* (United States). The gut of Guatemalan passalids also was a source for yeasts in the *Lodderomyces* clade that are closely related to the X-F yeasts, *C. tropicalis* and *C. parapsilosis* (Figs 3 and 4). Altogether, these findings suggest that the C-F and X-F yeasts in the clades *Scheffersomyces*, *Spathaspora* and *Lodderomyces* are common in the gut of lignicolous insects, especially passalids.

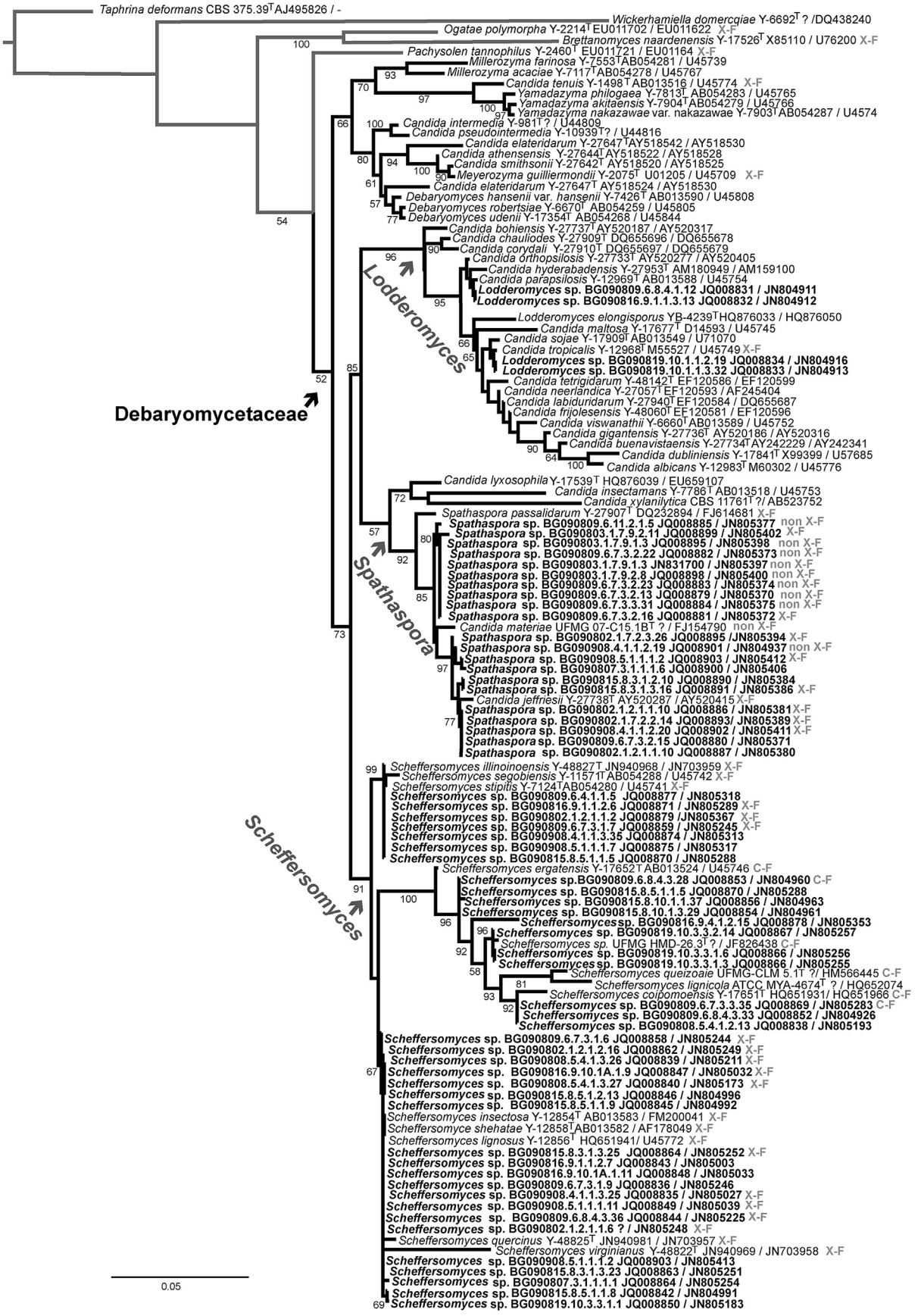

Fig 4 – Saccharomycotina yeast lineages associated with the gut of Guatemalan passalids with emphasis on the family Debaryomycetaceae in which most of the known X-F yeasts are classified. Maximum likelihood (ML) phylogenetic tree based on SSU and LSU loci. Results of the cellobiose (C-F) and xylose-fermenting (X-F) assays are indicated. Outgroups are shown in gray and strains isolated in this study are shown in bold. Final ML score: −16009.37.

Other ascomycete lineages associated with the gut of Guatemalan passalids

The gut of Guatemalan passalids was a source of several yeast strains in the *Sugiyamaella* clade (79 isolates, approximately 10.25 %) (Fig 5), two *Phaffomyces* strains (Phaffomycetaceae) (GenBank JN804920 and JN804921) and two *Spencermartinsiella* strains, all classified in Trichomonascaceae (Peter et al., 2011) (GenBank JN805442 and JN805443).

This study provides the first report of *Phaffomyces* species from the gut of wood-feeding beetles. Four yeast species in this genus previously were found in association with cactophilic *Drosophila* and necrotic wounds in cereoid cacti from Australia, North America and the Antilles (Kurtzman et al.,

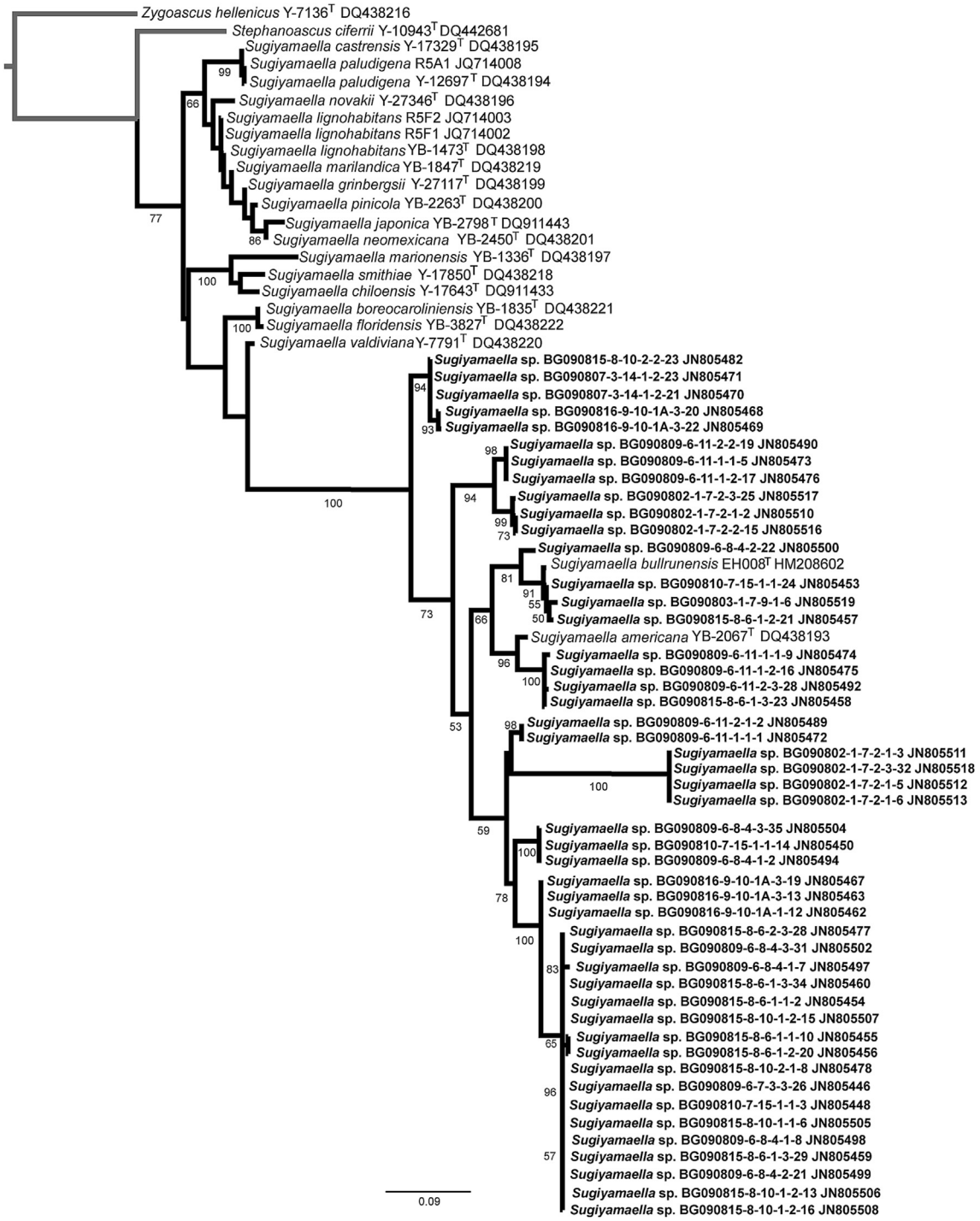

Fig 5 – *Sugiyamaella* species associated with the gut of Guatemalan passalids. Maximum likelihood phylogenetic tree based on LSU sequences. Note that most species isolated from passalids occur in a distinctive clade. Outgroups are shown in gray and strains isolated in this study are shown in bold. Final ML score: −3362.45.

2011). In addition to their ability to grow on lactate and succinate the use of ethanol as a sole carbon source is another physiological trait shared by all *Phaffomyces* species. Use of ethanol might be associated with a natural environment rich in this compound, such as the gut of insects where fermentations are carried out by symbiotic gut microbiota.

The *Sugiyamaella* clade is composed of 17 cosmopolitan species mainly isolated previously from softwood and hardwood trees and insects in the United States, Chile and Japan (Kurtzman, 2007; Houseknecht et al., 2011; Urbina et al., 2013) (Fig 5). The occurrence of members of this clade in the gut of lignicolous insects was confirmed previously by metagenomic studies (GenBank AY390773 and AY390774) (Zhang et al., 2003) and classical microbiological studies performed on longhorned beetles (Cerambycidae) (Grunwald et al., 2010), lignicolous insects (Houseknecht et al., 2011) and wood-roaches (*Cryptocercus* sp.) (Urbina et al., 2013).

In terms of physiological profiles, the *Sugiyamaella* members all assimilate D-xylose and L- and D-arabinose, although they are not able to ferment xylose (Kurtzman et al., 2011). In common with other members of this clade, the undescribed species of *Sugiyamaella* isolated from the gut of Guatemalan passalids did not ferment xylose (data not shown). The *Sugiyamaella* yeasts found in association with the Guatemalan passalids were related to *Sugiyamaella americana* and *S. bullrunensis*, previously isolated from frass of insects that feed on dead wood in the United States (Houseknecht et al., 2011) (Fig 5). The undescribed *Sugiyamaella* species isolated in Guatemala were associated exclusively with passalids in the subfamily Proculinae; although we isolated more than 164 yeast strains (~21 %) from passalids in the subfamily Passalinae, *Sugiyamaella* species were never present (Fig 3).

A key difference among passalids in Passalinae and Proculinae is their habitat. Beetles in Proculinae colonize rotted sapwood and heartwood, while members of Passalinae generally occupy a position just beneath the loose outer bark (Reyes-Castillo, 1970; Castillo and Lobo, 2004). The habitat difference also is reflected in the external morphology of the species because members of Proculinae possess bodies that are ovoid and Passalinae have flattened bodies in cross section (Castillo and Lobo, 2004). The shift in habitat is possibly a consequence of the feeding behavior and might help to explain the absence of *Sugiyamaella* in passalines (Fig 3). Houseknecht et al. (2011) showed a similar association based on the occasional presence of *S. bullrunensis* in the gut of *O. disjunctus*, another proculine species; they also mentioned that additional broad-scale samplings will be necessary to clarify the ecological relationships between *Sugiyamaella* and xylophagous insects. Overall, the occasional presence of *Phaffomyces*, *Spencermartinsiella* and *Sugiyamaella* in the gut of Guatemalan passalids may be attributable to geographical variation and/or substrate preferences of the passalid hosts of the yeasts. It is also important to note that rare yeasts may not colonize the gut but could be obtained occasionally from the substrate.

Basidiomycete yeasts associated with the gut of Guatemalan passalids

Species of basidiomycete yeasts in *Cryptococcus* and *Trichosporon* (Tremellomycetes, Agaricomycotina) were found consistently in the gut of Guatemalan passalids (60 isolates, 0.8 %) (Figs 3 and 6). In culture these basidiomycete yeasts exhibit a farinose colony morphology, which makes them easy to distinguish from other yeasts, a reason why we were able to obtain several isolates of the rare taxa. Recent studies have focused on the description and characterization of *Trichosporon* species in association with insects (Middelhoven et al., 2004; Molnar et al., 2004; Nakase et al., 2006; Fuentefria et al., 2008; Pagnocca et al., 2010; Gujjari et al., 2011). Specifically, *Trichosporon scarabaeorum* (Middelhoven et al., 2004), *T. insectorum* (Fuentefria et al., 2008) and *T. xylopini* (Gujjari et al., 2011) were first found associated with wood-feeding insects, and *Trichosporon siamense* and *Cryptococcus humicola* were isolated from the gut of passalids (*O. disjunctus*, *P. interstitialis*, *Passalus punctiger* and *V. hageni*) (Meredith Blackwell and Sung-Oui Suh, unpublished data).

The abilities to decompose and utilize wood components (e.g. cellulose and xylan) are characteristics of several *Trichosporon* species. The association of *Trichosporon* yeasts with soil plant debris and the gut of lignicolous insects supports a hypothesis that the yeasts may play a fundamental role in the decomposition and recycling of wood components in the ecosystem (Berkov et al., 2007; Gujjari et al., 2011). Basidiomycete yeasts in *Cryptococcus* and *Trichosporon*, therefore, may be involved in the decomposition of wood components, the feeding substrate of passalids.

Comparison of yeast diversity within gut regions

The X-F *S. shehatae* was always recovered from the three gut regions in the majority of the beetles studied. Although *S. shehatae* and other yeast species could be isolated from all gut regions (e.g. Fig 7), yeast diversity tended to increase in the PHG. No statistical difference, however, was found in species richness between gut regions and among passalid individuals (Chi-squared values: F–M = 3.36, $P = 0.76$; AHG = 7.62, $P = 0.27$; PHG = 3.43, $P = 0.91$) nor passalid species (Chi-squared values: F–M = 2.80, $P = 0.83$; AHG = 5.53, $P = 0.48$; PHG = 7.60, $P = 0.47$). Previous studies have reported that *S. stipitis* is capable of colonizing the PHG of *O. disjunctus* with filamentous growth attached by a holdfast (Lichtwardt et al., 1999; Suh et al., 2004b; Nardi et al., 2006), but it is not known if other yeast species have similar stages within the guts. The results suggesting that the species composition of the yeasts inhabiting the gut of passalids was the same in all the gut compartments was unexpected given the restricted PHG attachment of *O. disjunctus*. It is clear, however, that gut-inhabiting yeasts must possess adaptations that allow them to survive conditions present in the different gut regions, including low O_2 availability in the anterior hindgut and dramatic changes in pH, to colonize the PHG.

Symbiotic yeasts can survive various physiological conditions in the interior of the insect gut as they travel from the mouth to the site of colonization. In addition to the observations on the colonization of the PHG by *S. stipitis*, we know something about other xylophagous beetles that contain related X-F yeasts: (1) *S. shehatae* and *Candida rhagii* arrive at mycetosomes off the gut of the xylophagous beetles *Rhagium inquisitor* and *Leptura rubra* (Cerambycidae) (Grunwald et al., 2010); (2) yeasts were consistently isolated from the frass of

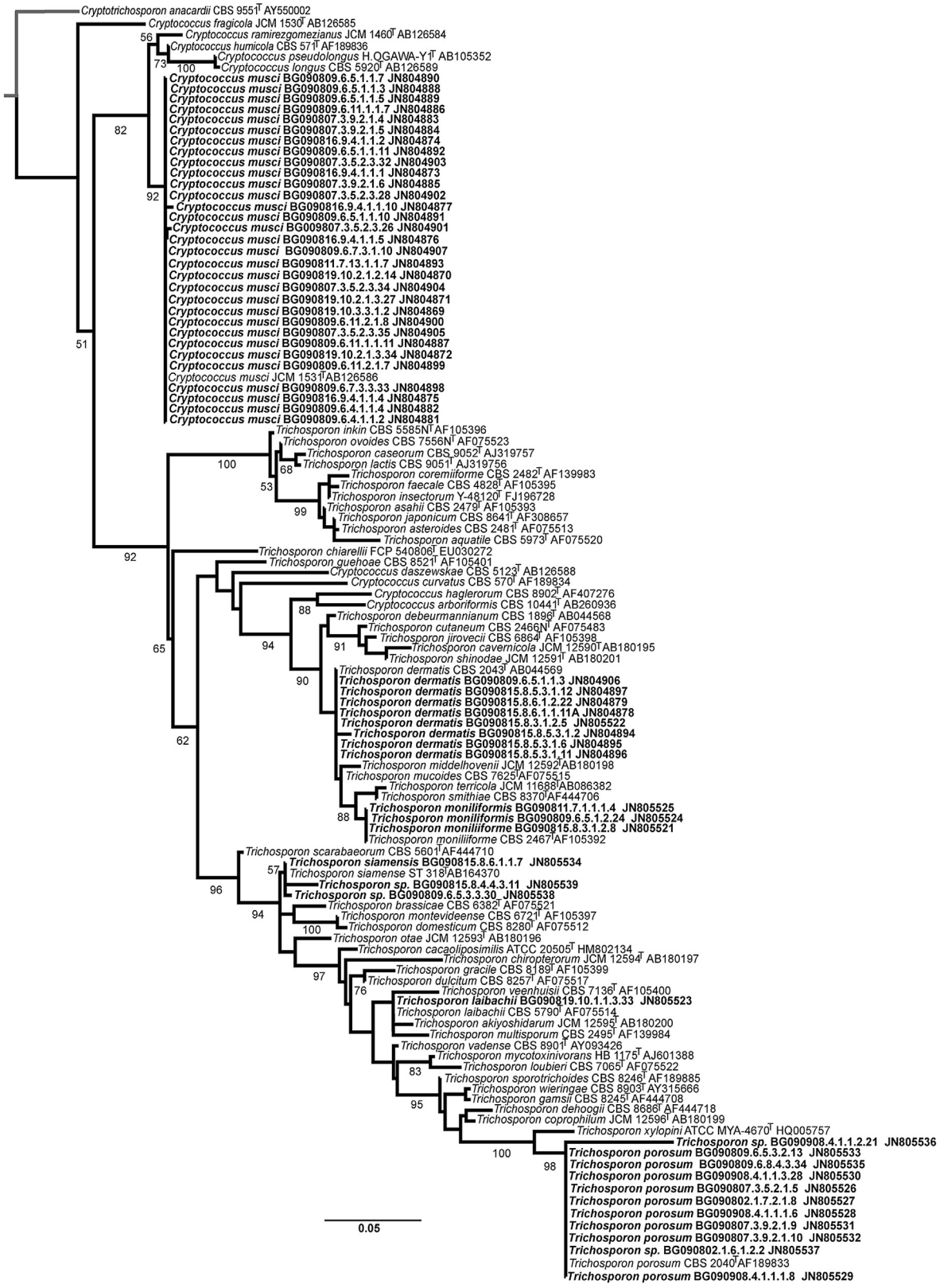

Fig 6 – *Cryptococcus* and *Trichosporon* species isolated from the gut of Guatemalan passalids. Maximum likelihood phylogenetic tree based on LSU sequences using *Cryptotrichoasporon anacardii* as an outgroup shown in gray and strains isolated in this study are shown in bold. Final ML score: −3671.43.

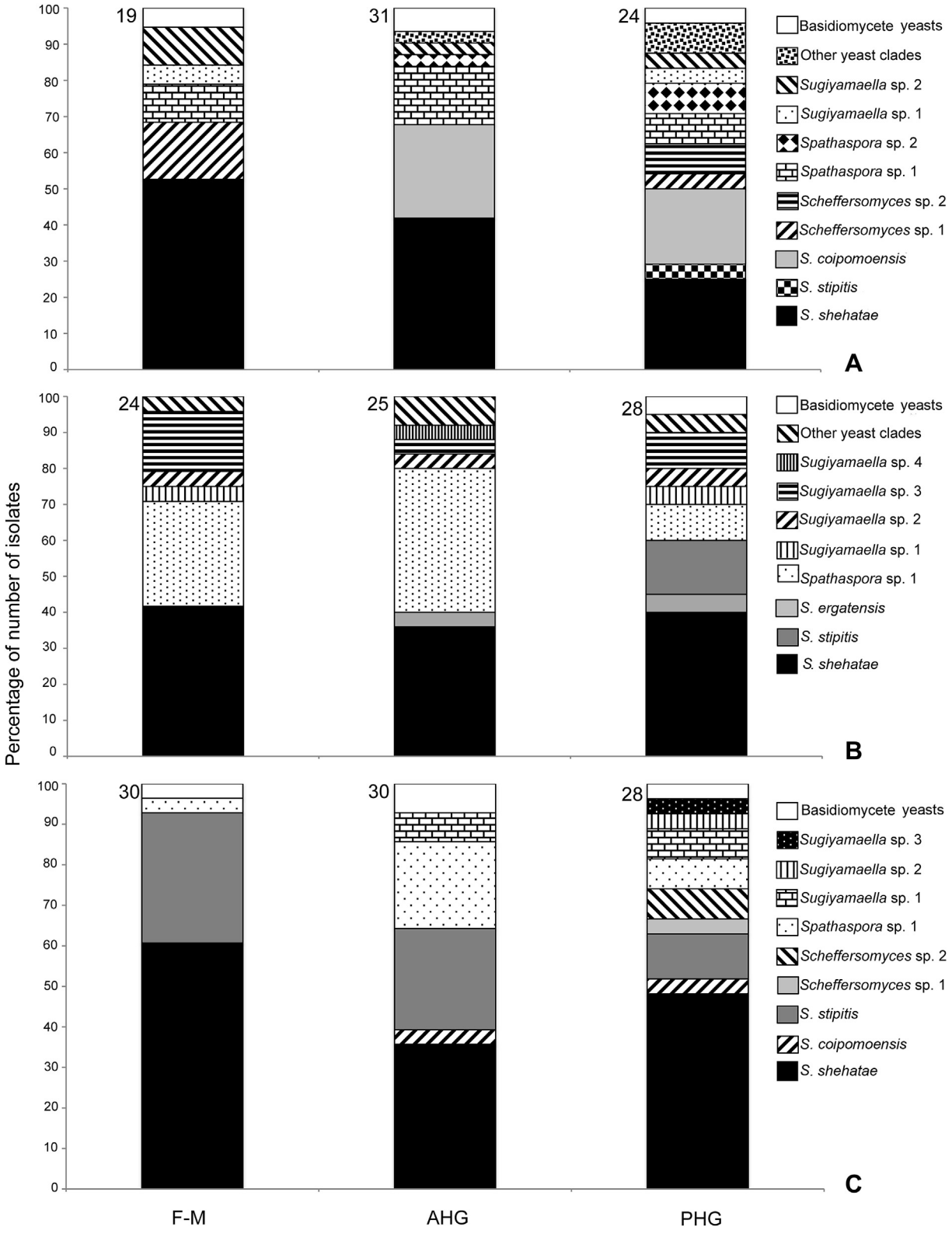

Fig 7 – Percentage of yeasts present in each gut region (F–M, Fore–midgut; AHG, anterior hindgut; PHG, posterior hindgut) of three passalid species: (A) *O. sargi*, (B) *C. debilis* and (C) *C. purulensis*. Notice that the X-F yeast *S. shehatae* was always present in all gut compartments. Other beetles showed the same pattern (data not shown). Total numbers of yeast isolates are indicated at the upper left corner of each column.

wood-feeding beetles [(e.g. *Candida endomychidarum*, *Yamadazyma* clade) from cerambycid beetles (Calderon and Berkov, 2012); *Candida thailandica* and *Scheffersomyces lignicola* from an undescribed insect (Jindamorakot et al., 2007)]; and (3) the behavior in which adults and larval passalids require feeding on pre-digested wood and frass coating their galleries (Reyes-Castillo, 1970; Boucher, 2005) to ensure horizontal transfer of microbes from parents to descendants. Yeasts also are able to survive external environmental conditions within the frass and wood (Cadete et al., 2009; Calderon and Berkov, 2012; Urbina and Blackwell, 2012). Additional studies, focused on the characterization of gut-inhabiting yeasts from lignicolous insects, could intensify the yeast sampling effort to include more host individuals by reducing the number of gut parts to be sampled and by concentrating on the PHG region colonized almost exclusively by yeasts, increasing the likelihood of selection of rare yeasts.

Many years ago gut morphology was suggested as a potential phylogenetically informative character in passalids (Reyes-Castillo, 1970). More recently, a hypothesis of evolutionary relationships among passalids was proposed based on gut morphology (Fonseca et al., 2011). The latter study described the AHG of Aulacocyclinae members as having none or few fermentation chambers and postulated the evolutionary changes including overall enlargement of the gut and addition of several fermentation chambers in members of the subfamilies Passalinae and Proculinae. Evidence for this is the microbial composition, primarily bacteria and archaea, in the AHG, fermentation of sugars and polymers from the substrate occurs in that compartment (Nardi et al., 2006; Huang et al., 2010; Ceja-Navarro et al., 2013). The evolutionary trend of the AHG from simple to complex may have been driven by the incorporation of a diversity of microbes to increase the breakdown and utilization of the polymers and sugars from wood. This hypothesis, however, has not been tested explicitly.

Comparison of the gut yeast community among host species

The yeast sampling recovered only 56 % of the estimated species richness (Fig 8 and 9). Based on the rarefaction curve, approximately 150 yeast species were predicted to occur in association with the 16 passalid species collected in Guatemala with an average number of 20 yeast species per host species (Table 3). An estimated 19 individual passalid species should be expected to yield 95 % of the total yeast species richness with a predicted 30 yeast species per host (Table 3). By extrapolation, based on an estimated worldwide total of 960 passalid species (Schuster, 2006), the yeast species richness should approach 6 500 species. If there is any degree of host specificity, these data imply that the yeast diversity of the gut of lignicolous insects remains very poorly understood. Host specificity, however, was not observed among the most abundant species and is an important factor in estimation of species richness that was not considered here.

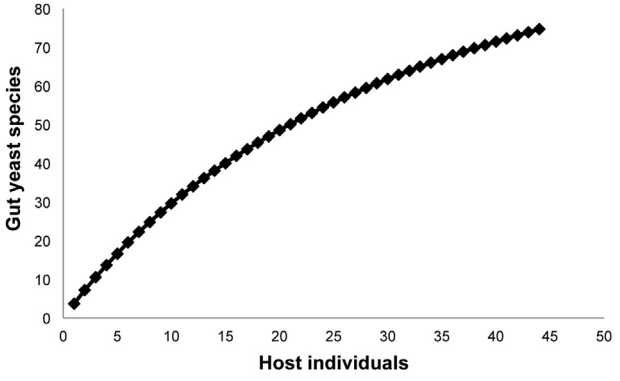

Fig 8 – Rarefaction curve by yeast species and beetle individual using a species richness matrix (list of increased rate at the beginning of the collection (a = 3.793) and curve shape value (b = 0.029), R^2 = 0.998).

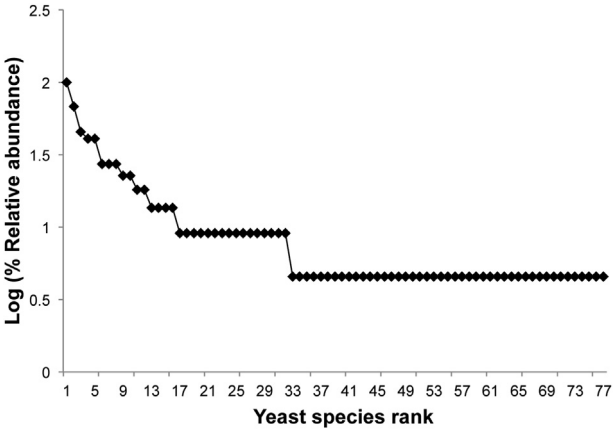

Fig 9 – Whittaker rank abundance curve. Forty-seven yeast species (57 % of the total yeast species richness) were recovered from single passalids.

Yeast species richness was influenced by the species of the host and substrate (Table 3). Differences in yeast species composition among individual beetles of the same species did occur, however, due to occasional rare species isolated from only one or two individuals (Fig 9). Based on the results of the forward selection analysis, the variables that most affect the yeast species richness are host length (R^2 = 0.083, P = 0.0017) and host species (R^2 = 0.063, P = 0.015), but these variables explain only 15 % of the variance. The lack of explanatory power for the variables used could be attributed to the fact that all beetles studied were adults that feed on rotten hardwoods in the same localities and sometimes in the same log, increasing the chance of horizontal transmission of the same symbionts. Therefore, other factors, not measured in this study may have strong influence on yeast species variation, such as the broad range of decomposing substrates in which passalids feed. Besides rotting hardwoods, certain passalids are able to feed on rotting monocotyledonous angiosperms (palms) and, less frequently, gymnosperms (conifers); *P. angulatus* lives in the disposal waste chambers of *Atta* ants (Castillo and Lobo, 2004; Schuster, 2006). Although we noticed a preference for dead white-rotted hardwoods, some species were collected inhabiting only recently dead logs and other species, only in logs in an advanced stage of decomposition; the depletion and concentration of components in decayed wood varies during the decomposition process depending on the decomposer organisms (Kirk and Cowling, 1984). In addition ontogeny of the

insects during metamorphosis from larvae to adults provides new, more complex cuticular landscapes in the gut (Nardi et al., 2006), increasing the chance of a concomitant change in microbiota. These results will help to refine further studies in order to understand what factors have strong influence on the gut-inhabiting yeast communities.

The results presented here are in agreement with previous studies focused on the characterization of the yeasts present in the gut of the temperate North American passalid, *O. disjunctus*, and a few other passalid species. Most studies indicate that the yeast clades found in association with *O. disjunctus* (*Cryptococcus*, *Kazachstania*, *Scheffersomyces*, *Spathaspora*, *Sugiyamaella* and *Trichosporon*) have similar associations among Guatemalan passalids (Zhang et al., 2003; Suh et al., 2004b; Nardi et al., 2006; Gujjari et al., 2011; Houseknecht et al., 2011; Suh and Zhou, 2011). The one exception was members of the *Kazachstania* clade, which were not isolated from Guatemalan passalids, and yeasts in the *Phaffomyces* and *Spencermartinsiella* clades, which were found only in association with Guatemalan passalids.

Importance of the yeast gut community in the host biology

The diversity of gut-inhabiting bacteria from certain wood-feeding insects such as termites and roaches is well known, and the morphological characterization of the symbiotic relationships shows a close connection between host and bacterial cells. The associations between fungi and insects are less well known, but some studies suggest that digestive enzymes and other microbial products (e.g., essential amino acids, vitamins and sterols) provided by yeasts are essential to the insect host (Suh et al., 2001, 2003; Vega and Dowd, 2005). Yeasts also may play an important role in the detoxification of plant metabolites in the diet of the hosts, and fermentation in the gut may increase the nutritional quality of the insect food materials (Steinkraus, 1994). The microbial decomposition of cellulose provides intermediate products such as cellobiose and glucose that can be directly used by the host insect or further used as substrate for fermentation by yeasts. During alcoholic fermentations O_2 is consumed and CO_2 is produced enhancing an anaerobic environment for the bacterial community. The expression of fungal xylanases in the gut of wood-feeding insects has been used as evidence to support that the decomposition of wood polymers is carried out within the insect gut by fungi (Brennan et al., 2004).

The degree to which passalids benefit from the association with yeasts, however, is not clear because studies of gut absorbance in a variety of insects have indicated that uptake from the posterior part of the hindgut is limited to water and some salts (Gullan and Cranston, 2005). *S. stipitis* has been observed attached only to the PHG cuticle (Lichtwardt et al., 1999; Nardi et al., 2006); although it is assumed to be metabolically active in this location, this and other yeast species occur in all gut compartments where their metabolic activity is not known (Fig 7). Perhaps yeasts benefit preferentially from the association in which they "come in from the cold" to enjoy an environment rich in organic compounds as potential nutrients; in addition the yeasts likely acquire an increased dispersal range and a protected habitat (Vega and Dowd, 2005).

Future prospects

The consistent and dominant presence of the X-F yeasts *S. shehatae* and *S. stipitis* and the poor morphological differences among yeast colonies hinder the study of the total diversity of gut-inhabiting yeasts by classic microbiological techniques. Our yeast sampling methodology allowed for the isolation and identification of gut-inhabiting yeasts by exhaustively selecting yeast colonies using passalid individuals as sampling units. We isolated several undescribed species, which will inform the phylogenetic relationships of yeasts. The application of next-generation sequencing in the future will increase accuracy in the estimation, comparison and dynamics of the gut-inhabiting yeasts associated with wood-feeding beetles, although such studies will not provide the cultures that are indispensable for our understanding of the phylogenetic relationship among yeasts.

Xylose-fermenting yeasts may have great potential in the biotechnology industry due to their ability to decompose and ferment wood components, so by using selective media containing a pentose sugar (e.g. xylose, ribose, etc.) as a sole source of carbon and energy, could help to increase the chance of isolation of yeasts that efficiently decompose certain pentoses. The application of this methodology among passalids present in Africa, Asia and Australia, and across different families of lignicolous beetles would help to elucidate the dynamics of yeast composition associated with wood-feeding arthropods. The isolation of several strains of the same species from different hosts would provide a generic resource that can be used for the study of genetic variation among yeasts.

Acknowledgments

We thank Louisiana State University students Robert Frank, Simone Mack, Juan Herrera, Matthew Knight, Elizabeth Cooper, and Stephanie Gross for their skilled assistance in all phases of this study. We thank Diego Pons, Enio Cano and Christian Beza for their support during the fieldwork. Sandra Galeano, Katherine Hovanes, James Cronin, Kyle Harms and Maria Mercedes Gavilanez helped with the statistical analyses. This work was supported by the National Science Foundation grants NSF-DEB-0417180 and NSF-0732671, and subcontract DE-AC52-07NA27344 from Lawrence Livermore National Laboratory.

REFERENCES

Adam, H., Groenewald, M., Mohan, S., et al., 2009. Identification of a new species, *Candida subhashii*, as a cause of peritonitis. Medical Mycology 47, 305–311.

Barbosa, A.C., Cadete, R.M., Gomes, F.C., et al., 2009. *Candida materiae* sp. nov., a yeast species isolated from rotting wood in the Atlantic Rain Forest. Journal of Systematic and Evolutionary Microbiology 59, 2104–2106.

Barnett, J.A., Payne, R.W., Yarrow, D., 2000. Yeasts: Characteristics and Identification, third ed. Cambridge University Press, Cambridge.

Berkov, A., Feinstein, J., Small, J., Nkamany, M., 2007. Yeasts isolated from form neotropical wood-boring beetles in SE Peru. Biotropica 39, 530–538.
Boonmak, C., Limtong, S., Jindamorakot, S., et al., 2011. *Candida xylanilytica* sp nov., a xylan-degrading yeast species isolated from Thailand. International Journal of Systematic and Evolutionary Microbiology 61, 1230–1234.
Boucher, S., 2005. Évolution et phylogénie des Coléoptères Passalidae (Scarabaeoidea). Annales de la Société Entomologique de France 41, 239–604.
Brennan, Y., Callen, W.N., Christoffersen, L., et al., 2004. Unusual microbial xylanases from insect guts. Applied and Environmental Microbiology 70, 3609–3617.
Bryan, J.H.D., 1954. Cytological and cytochemical studies of oogenesis of *Popilius disjunctus* Illiger (Coleoptera, Polyphaga). Biological Bulletin 107, 64–79.
Buchler, E.R., Wright, T.B., Brown, E.D., 1981. On the functions of stridulation by the passalid beetle *Odontotaenius disjunctus* (Coleoptera, Passalidae). Animal Behaviour 29, 483–486.
Burnett, A.M., Mason, W.H., Rhodes, S.T., 1969. Reingestion of feces and excretion rates of Zn-65 in *Popilius disjunctus* versus *Cryptocercus punctulatus*. Ecology 50, 1094–1096.
Cadete, R.M., Santos, R.O., Melo, M.A., et al., 2009. *Spathaspora arborariae* sp. nov., a D-xylose–fermenting yeast species isolated from rotting wood in Brazil. FEMS Yeast Research 9, 1338–1342.
Calderon, O., Berkov, A., 2012. Midgut and fat body bacteriocytes in neotropical cerambycid beetles (Coleoptera: Cerambycidae). Environmental Entomology 41, 108–117.
Castillo, M.L., Lobo, J.M., 2004. A comparison of Passalidae (Coleoptera, Lamellicornia) diversity and community structure between primary and secondary tropical forest in Los Tuxtlas, Veracruz, Mexico. Biodiversity and Conservation 13, 1257–1269.
Ceja-Navarro, J.A., Nguyen, N.H., Karaoz, U., Gross, S.R., Herman, D.J., Andersen, G.L., Bruns, T.D., Pett-Ridge, J., Blackwell, M., Brodie, E.L., 2013. Compartmentalized microbial composition, oxygen gradients and nitrogen fixation in the gut of *Odontotaenius disjunctus*. The ISME Journal. in press.
Chang, C.F., Yao, C.H., Young, S.S., et al., 2011. *Candida gosingica* sp. nov., an anamorphic ascomycetous yeast closely related to *Scheffersomyces spartinae*. International Journal of Systematic and Evolutionary Microbiology 61, 690–694.
Chao, A., 1987. Estimating the population size for capture-recapture data with unequal catchability. Biometrics 43, 783–791.
Collings, S.B., 1966. An improved ringer solution for heart of *Passalus* beetle *Popilius disjunctus* (Coleoptera: Passalidae). Annals of the Entomological Society of America 59, 972–974.
Colwell, R.K., 2006. EstimateS: Statistical Estimation of Species Richness and Shared Species from Samples. Version 8. http://viceroy.eeb.uconn.edu/estimates/index.html.
Delfinado, M.D., Baker, E.W., 1975. Mites (Acarina) associated with *Popilius disjunctus* (Illiger) (Coleoptera: Passalidae) in Eastern United States. Journal of the New York Entomological Society 83, 49–59.
Dismukes, J.F., Mason, W.H., 1975. Effects of carbaryl and methyl parathion poisoning on bioelimination of Zn-65 and Mn-54 in *Popilius disjunctus* Coleoptera-Passalidae. Environmental Entomology 4, 221–224.
Ferguson, D.E., Land, J.D., 1961. Some temperature studies on beetle, *Popilius disjunctus*. Ecology 42, 195–197.
Fonseca, C.R., Barbosa, M.L., Fernandez, M.F., 2011. A hypothetical evolutionary history of passalid beetles narrated by the comparative anatomy of the hindgut (Coleoptera: Passalidae). Zootaxa, 1–20.
Fuentefria, A.M., Suh, S.O., Landell, M.F., et al., 2008. *Trichosporon insectorum* sp. nov., a new anamorphic basidiomycetous killer yeast. Mycological Research 112, 93–99.
Gibson, G.E., 1977. Seasonal distribution of mites associated with *Popilius disjunctus* (Illiger) (Coleoptera-Passalidae) in Hardin County, Texas. Texas Journal of Science 28, 109–112.
Gotelli, N.J., Colwell, R.K., 2001. Quantifying biodiversity: procedures and pitfalls in the measurement and comparison of species richness. Ecology Letters 4, 379–391.
Grunwald, S., Pilhofer, M., Holl, W., 2010. Microbial associations in gut systems of wood- and bark-inhabiting longhorned beetles [Coleoptera: Cerambycidae]. Systematic and Applied Microbiology 33, 25–34.
Gujjari, P., Suh, S.O., Lee, C.F., Zhou, J.L.J., 2011. *Trichosporon xylopini* sp nov., a hemicellulose-degrading yeast isolated from the wood-inhabiting beetle *Xylopinus saperdioides*. International Journal of Systematic and Evolutionary Microbiology 61, 2538–2542.
Gullan, P.J., Cranston, P.S., 2005. The Insects: an Outline of Entomology, thrid ed. Blackwell Publishing, Oxford. pp. xvii + 505 + 6 plates.
Hiznay, P.A., Krause, J.B., 1955. The structure and musculature of the larval head and mouthparts of the horned *Passalus* beetle, *Popilius disjunctus* Illiger. Journal of Morphology 97, 55–75.
Houseknecht, J.L., Hart, E.L., Suh, S.O., Zhou, J.J., 2011. Yeasts in the *Sugiyamaella* clade associated with wood-ingesting beetles and the proposal of *Candida bullrunensis* sp. nov. International Journal of Systematic and Evolutionary Microbiology 61, 1751–1756.
Huang, S.W., Zhang, H.Y., Marshall, S., Jackson, T.A., 2010. The scarab gut: a potential bioreactor for bio-fuel production. Insect Science 17, 175–183.
Jackson, H.B., Baum, K.A., Robert, T., Cronin, J.T., 2009. Habitat-specific movement and edge-mediated behavior of the saproxylic insect *Odontotaenius disjunctus* (Coleoptera: Passalidae). Environmental Entomology 38, 1411–1422.
Jimenez-Valverde, A., Hortal, J., 2003. Las curvas de acumulacion de especies y la necesidad de evaluar la calidad de los inventarios biologicos. Revista Iberica de Aracnologia 8, 151–161.
Jindamorakot, S., Limtong, S., Yongmanitchai, W., et al., 2007. Two new anamorphic yeasts, *Candida thailandica* sp. nov. and *Candida lignicola* sp. nov., isolated from insect frass in Thailand. FEMS Yeast Research 7, 1409–1414.
King, A., Fashing, N., 2007. Infanticidal behavior in the subsocial beetle *Odontotaenius disjunctus* (Illiger) (Coleoptera: Passalidae). Journal of Insect Behavior 20, 527–536.
Kirk, T., Cowling, E., 1984. Biological decomposition of solid wood. In: Roger, R.M. (Ed.), The Chemistry of Soil Wood. American Chemical Society, Washington, DC, pp. 455–487. (Chapter 12).
Kurtzman, C.P., 1990. *Candida shehatae*, genetic diversity and phylogenetic relationships with other xylose-fermenting yeasts. Antonie van Leeuwenhoek 57, 215–222.
Kurtzman, C.P., 2007. Eleven new species of *Sugiyamaella* and *Candida* from forest habitats. FEMS Yeast Research 7, 1046–1063.
Kurtzman, C.P., Fell, J.W., Boekhout, T., 2011. The Yeasts, A Taxonomic Study. Elsevier, Amsterdam. pp. xxii + 1–289, xv + 290–1335, xvi + 1336–2080.
Kurtzman, C.P., Robnett, C.J., 1998. Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Antonie van Leeuwenhoek 73, 331–371.
Kurtzman, C.P., Robnett, C.J., 2007. Multigene phylogenetic analysis of the *Trichomonascus, Wickerhamiella* and *Zygoascus* yeast clades, and the proposal of *Sugiyamaella* gen. nov. and 14 new species combinations. FEMS Yeast Research 7, 141–151.
Kurtzman, C.P., Suzuki, M., 2010. Phylogenetic analysis of ascomycete yeasts that form coenzyme Q-9 and the proposal of the new genera *Babjeviella, Meyerozyma, Millerozyma, Priceomyces*, and *Scheffersomyces*. Mycoscience 51, 2–14.

Lichtwardt, R.W., White, M.M., Cafaro, M.J., Misra, J.K., 1999. Fungi associated with passalid beetles and their mites. Mycologia 91, 694–702.
Lin, Y., He, P., Wang, Q., et al., 2010. The alcohol dehydrogenase system in the xylose-fermenting yeast *Candida maltosa*. PLoS One 5, e11752.
Liu, K., Warnow, T.J., Holder, M.T., et al., 2012. SATe-II: very fast and accurate simultaneous estimation of multiple sequence alignments and phylogenetic trees. Systematic Biology 61, 90–106.
Lohmeier-Vogel, E., Skoog, K., Vogel, H., Hahn-Hagerdal, B., 1989. 31P nuclear magnetic resonance study of the effect of azide on xylose fermentation by *Candida tropicalis*. Applied and Environmental Microbiology 55, 1974–1980.
MacGown, J., MacGown, M., 1996. Observation of a nuptial flight of the horned passalus beetle, *Odontotaenius disjunctus* (Illiger) (Coleoptera: Passalidae). Coleopterists Bulletin 50, 201–203.
Maddison, W., Maddison, D., 2005. Mesquite: a modular system for evolutionary analysis. Evolution 62, 1103–1118.
Mason, W.H., Wit, L.C., Blackmore, M.S., 1983. Bioelimination of Zn-65 in *Popilius disjunctus* after a dietary zinc supplement. Journal of the Georgia Entomological Society 18, 246–251.
Middelhoven, W.J., Scorzetti, G., Fell, J.W., 2004. Systematics of the anamorphic basidiomycetous yeast genus *Trichosporon* Behrend with the description of five novel species: *Trichosporon vadense*, *T. smithiae*, *T. dehoogii*, *T. scarabaeorum* and *T. gamsii*. International Journal of Systematic and Evolutionary Microbiology 54, 975–986.
Molnar, O., Schatzmayr, G., Fuchs, E., Prillinger, H., 2004. *Trichosporon mycotoxinivorans* sp. nov., a new yeast species useful in biological detoxification of various mycotoxins. Systematic and Applied Microbiology 27, 661–671.
Nakase, T., Jindamorakot, S., Sugita, T., et al., 2006. *Trichosporon siamense* sp. nov. isolated from insect frass in Thailand. Mycoscience 47, 106–109.
Nardi, J.B., Bee, C.M., Miller, L.A., et al., 2006. Communities of microbes that inhabit the changing hindgut landscape of a subsocial beetle. Arthropod Structure and Development 35, 57–68.
Nguyen, N.H., Suh, S.O., Marshall, C.J., Blackwell, M., 2006. Morphological and ecological similarities: wood-boring beetles associated with novel xylose-fermenting yeasts, *Spathaspora passalidarum* gen. sp. nov. and *Candida jeffriesii* sp. nov. Mycological Research 110, 1232–1241.
Oksanen, J., Blanchet, F., Kindt, R., et al., 2012. vegan: Community Ecology Package. R Package.
Pagnocca, F.C., Legaspe, M.F., Rodrigues, A., et al., 2010. Yeasts isolated from a fungus-growing ant nest, including the description of *Trichosporon chiarellii* sp. nov., an anamorphic basidiomycetous yeast. International Journal of Systematic and Evolutionary Microbiology 60, 1454–1459.
Pearse, A.S., Patterson, M.T., Rankin, J.S., Warton, G.W., 1936. The ecology of *Passalus cornutus* Fabricius, a beetle which lives in rotting logs. Ecological Monographs 6, 455–490.
Peter, G., Dlauchy, D., Tornai-Lehoczki, J., et al., 2011. *Spencermartinsiella europaea* gen. nov., sp. nov., a new member of the family Trichomonascaceae. International Journal of Systematic and Evolutionary Microbiology 61, 993–1000.
Punzo, F., 2007. Social facilitation and digging behavior in the beetle *Odontotaenius floridanus* Schuster (Coleoptera: Passalidae). Journal of Entomological Science 42, 525–532.
R Development Core Team, 2005. R: a Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
Rains, T.D., Dimock, R.V., 1978. Seasonal-variation in cold hardiness of beetle *Popilius disjunctus*. Journal of Insect Physiology 24, 551–554.
Rao, R.S., Bhadra, B., Shivaji, S., 2007. Isolation and characterization of xylitol-producing yeasts from the gut of colleopteran insects. Current Microbiology 55, 441–446.
Reyes-Castillo, P., 1970. Coleoptera: Passalidae; morfologia y division en grandes grupos; generos americanos. Folia Entomologica Mexicana 20–22, 1–240.
Rivera, F.N., Gonzalez, E., Gomez, Z., et al., 2009. Gut-associated yeast in bark beetles of the genus *Dendroctonus* Erichson (Coleoptera: Curculionidae: Scolytinae). Biological Journal of the Linnean Society 98, 325–342.
Robbertse, B., Reeves, J.B., Schoch, C.L., Spatafora, J.W., 2006. A phylogenomic analysis of the Ascomycota. Fungal Genetics and Biology 43, 715–725.
Roberts, H.S., 1952. The meiotic spindle of *Popilius disjunctus*. Anatomical Record 113, 538–539.
Robertson, C.H., 1962. The anatomy of the respiratory system of the *Passalus* beetles, *Popilius disjunctus* (Illiger). American Midland Naturalist 68, 376–393.
Santos, R.O., Cadete, R.M., Badotti, F., et al., 2011. *Candida queiroziae* sp. nov., a cellobiose-fermenting yeast species isolated from rotting wood in Atlantic Rain Forest. Antonie van Leeuwenhoek 99, 635–642.
Sawvel, J.W., Gunderson, S., Schiavone, R.C., Nelson, E.V., 1992. A method for thin sectioning heavily sclerotized cuticle of *Odontotaenius disjunctus*. Biotechnic & Histochemistry 67, 309–312.
Schoch, C.L., Seifert, K.A., Huhndorf, S., et al., 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for *Fungi*. Proceedings of the National Academy of Sciences of the United States of America 109, 6241–6246.
Schoch, C.L., Sung, G.H., Lopez-Giraldez, F., et al., 2009. The Ascomycota tree of life: a phylum-wide phylogeny clarifies the origin and evolution of fundamental reproductive and ecological traits. Systematic Biology 58, 224–239.
Schuster, J., 1975. A comparative study of copulation in Passalidae (Coleoptera): new positions for beetles. The Coleopterists Bulletin 29, 75–81.
Schuster, J., 1991. *Heliscus* and *Verres* (Coleoptera: Passalidae): new species records from Guatemala and Panama. Flora Entomologica 74, 475–476.
Schuster, J., 1992a. Arthropod collecting and forest conservation in Guatemala. Insect Collection News 8, 15–16.
Schuster, J., 1992b. Insect collections and biodiversity in Guatemala. Association Systematics Collections Newsletter 20, 90–91.
Schuster, J., 1993. *Xylopassaloides* (Coleoptera: Passalidae) in Mesoamerica: Relations, distribution and new species. Florida Entomologist 76, 114–119.
Schuster, J., 2002. Passalidae Leach 1815. Bess beetle family. In: Arnett, R.H., Thomas, M.C., Skekkey, P.E., Frank, J.H. (Eds.), American Beetles. Polyphaga: Scarabaeoidea through Curculionoidea. CRC Press, Boca Ratón, pp. 12–14.
Schuster, J., 2006. Passalidae (Coleoptera) de Mesoamerica: Diversidad y biogeografia. In: Cano, E. (Ed.), Biodiversidad de Guatemala. Universididad del Valle de Guatemala, Guatemala City, pp. 379–401.
Schuster, J., Cano, E., Cardona, C., 2000. Un método sencillo para priorizar la conservación de los bosques nubosos de Guatemala usando Passalidae (Coleoptera) como organismos indicadores. Acta Zoologica Mexicana 80, 197–209.
Schuster, J., Cano, E., Reyes-Castillo, P., 2003. *Proculus*, giant Latin-American Passalids: revision, phylogeny and biogeography. Acta Zoologica Mexicana 90, 281–306.
Soberon, J., Llorente, J., 1993. The use of species accumulation functions for the prediction of species richness. Conservation Biology 7, 480–488.

Stamatakis, A., 2006. RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22, 2688–2690.
Steinkraus, K.H., 1994. Nutritional significance of fermented foods. Food Research International 27, 259–267.
Suh, S.O., Blackwell, M., 2004. Three new beetle-associated yeast species in the *Pichia guilliermondii* clade. FEMS Yeast Research 5, 87–95.
Suh, S.O., Blackwell, M., 2005. Four new yeasts in the *Candida mesenterica* clade associated with basidiocarp-feeding beetles. Mycologia 97, 167–177.
Suh, S.O., Blackwell, M., 2006. Three new asexual arthroconidial yeasts, *Geotrichum carabidarum* sp. nov., *Geotrichum histeridarum* sp. nov., and *Geotrichum cucujoidarum* sp. nov., isolated from the gut of insects. Mycological Research 110, 220–228.
Suh, S.O., Marshall, C.J., McHugh, J.V., Blackwell, M., 2003. Wood ingestion by passalid beetles in the presence of xylose-fermenting gut yeasts. Molecular Ecology 12, 3137–3145.
Suh, S.O., McHugh, J.V., Blackwell, M., 2004a. Expansion of the *Candida tanzawaensis* yeast clade: 16 novel *Candida* species from basidiocarp-feeding beetles. International Journal of Systematic and Evolutionary Microbiology 54, 2409–2429.
Suh, S.O., White, M.M., Nguyen, N.H., Blackwell, M., 2004b. The status and characterization of *Enteroramus dimorphus*: a xylose-fermenting yeast attached to the gut of beetles. Mycologia 96, 756–760.
Suh, S.O., McHugh, J.V., Pollock, D.D., Blackwell, M., 2005a. The beetle gut: a hyperdiverse source of novel yeasts. Mycological Research 109, 261–265.
Suh, S.O., Nguyen, N.H., Blackwell, M., 2005b. Nine new *Candida* species near *C. membranifaciens* isolated from insects. Mycological Research 109, 1045–1056.
Suh, S.O., Nguyen, N.H., Blackwell, M., 2006. A yeast clade near *Candida kruisii* uncovered: nine novel *Candida* species associated with basidioma-feeding beetles. Mycological Research 110, 1379–1394.
Suh, S.O., Nguyen, N.H., Blackwell, M., 2008. Yeasts isolated from plant-associated beetles and other insects: seven novel *Candida* species near *Candida albicans*. FEMS Yeast Research 8, 88–102.
Suh, S.O., Noda, H., Blackwell, M., 2001. Insect symbiosis: derivation of yeast-like endosymbionts within an entomopathogenic filamentous lineage. Molecular Biology and Evolution 18, 995–1000.
Suh, S.O., Zhou, J.J., 2011. *Kazachstania intestinalis* sp nov., an ascosporogenous yeast from the gut of passalid beetle *Odontotaenius disjunctus*. Antonie van Leeuwenhoek International 100, 109–115.
Tafuri, J.F., Tafuri, F.X., 1983. Sex determination of *Popilius disjunctus* Illiger from body and horn dimensions. Ohio Journal of Science 83, 2.
Tallamy, D.W., Wood, T.K., 1986. Convergence patterns in subsocial insects. Annual Review of Entomology 31, 369–390.
Tanahashi, M., Kubota, K., Matsushita, N., Togashi, K., 2010. Discovery of mycangia and the associated xylose-fermenting yeasts in stag beetles (Coleoptera: Lucanidae). Naturwissenschaften 97, 311–317.
Unterseher, M., Schnittler, M., Dormann, C., Sickert, A., 2008. Application of species richness estimators for the assessment of fungal diversity. FEMS Microbiology Letters 282, 205–213.
Urbina, H., Blackwell, M., 2012. Multilocus phylogenetic study of the *Scheffersomyces* yeast clade and characterization of the N-terminal region of xylose reductase gene. PLoS One 7, e39128.
Urbina, H., Frank, R., Blackwell, M., 2013. *Scheffersomyces cryptocercus*: a new xylose–fermerting yeast associated with the gut of the wood roach and taxonomyc revision of the *Sugiyamaella* yeast clade. Mycologia 105, 650–660.
van der Walt, J.P., Ferreira, N.P., Steyn, R.L., 1987. *Candida lyxosophila* sp. nov., a new D-xylose fermenting yeast from southern Africa. Antonie van Leeuwenhoek 53, 93–97.
van der Walt, J.P., Scott, D.B., van der Klift, W.C., 1972. Six new *Candida* species form South Africa insect sources. Mycophathologia et Mycologia Applicata 47, 221–236.
Vega, F.E., Dowd, P.F., 2005. The role of yeasts as insect endosymbionts. In: Vega, F.E., Blackwell, M. (Eds.), Insect-fungal Associations, Ecology and Evolution. Oxford University Press, New York, pp. 211–243.
Ward, S.C., 1971. Carbon dioxide anesthesia of heart of horned passalus beetle, *Popilius disjunctus* Coleoptera-Passalidae. Annals of the Entomological Society of America 64, 430–437.
Wicknick, J.A., Miskelly, S.A., 2009. Behavioral interactions between non-cohabiting bess beetles, *Odontotaenius disjunctus* (Illiger) (Coleoptera: Passalidae). Coleopterists Bulletin 63, 108–116.
Wit, L.C., Mason, W.H., Blackmore, M.S., 1984. The effects of crowding on the bioelimination of Zn-65 in *Popilius disjunctus*. Journal of the Georgia Entomological Society 19, 8–14.
Yarrow, D., 1998. Methods for the isolation, maintenance and identification of yeasts. In: Kurtzman, C.P., Fell, J.W. (Eds.), The Yeasts, A Taxonomic Study. Elsevier, Amsterdam, pp. 77–100.
Zhang, N., Suh, S.O., Blackwell, M., 2003. Microorganisms in the gut of beetles: evidence from molecular cloning. Journal of Invertebrate Pathology 84, 226–233.